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Racial features in the heat tolerance limits in the silkworm, *Bombyx mori*

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Abstract: Temperature tolerance of 5th instar larvae and pupae of three races of the silkworm, *Bombyx mori* at 35, 38 and 40℃ was studied. LT_{50} values and feducial limits were determined in each case by Probit analysis. The multivoltine Pure Mysore showed better survival to high temperature treatment than the two bivoltine races. NB4D2 showed better temperature tolerance than CSR2 in the two bivoltine races. The 90, 70 and 29 kDa heat shock protein bands appeared in the haemolymph after exposure of the larvae to 38℃ for 6 h and to 40℃ for 3 h. The 29 kDa protein band could not be detected in the haemolymph of bivoltine larvae during recovery. But, expression of the 29 kDa protein was sustained during recovery in the larvae of Pure Mysore. When pupae were held at high temperature, the 90 and 70 kDa proteins were expressed, while the 29 kDa protein was not detected in the haemolymph. These results suggest that heat shock protein expression is associated with the occurrence of thermotolerance among tropical silkworm breeds, and growth stages within a breed.

Key words: *Bombyx mori*; race; haemolymph; heat shock proteins; thermotolerance

1 INTRODUCTION

Insects are enormously vulnerable to high temperature injury (Worner, 1998). Thermotolerance plays an important role in determining seasonal abundance and geographical distribution of the pea leaf miner, *Liriomyza huidobrensis* (Chen and Kang, 2002, 2005). Voltinism in the mulberry silkworm, *Bombyx mori* appears to have evolved in relation to the temperature characteristics of the regions of its abundance. The voltine character of *Bombyx mandarina*, the probable wild ancestor of the domesticated silkworm, *B. mori* is not clear. Bivoltine and multivoltine races of *B. mori* are characteristically present in temperate and tropical regions, respectively. Bivoltine races are also reared in tropical climates because of the higher silk content of their cocoons. There are no studies on the temperature tolerance of bivoltine and multivoltine races which are acclimated to cold and hot climates, respectively. Heat from the sun or artificial heat can quickly elevate body temperature in these small-bodied poikilotherms to lethal levels. The disadvantageous temperatures can severely impair normal growth and development in any insect. Mild heat hardening (4 h exposure to 32 or 35℃) significantly increased heat resistance of adult leaf miners, but it was unable to enhance cold survival, and cold hardening did not increase heat resistance (Huang *et al.*, 2007). Heat hardening increased cold tolerance but cold hardening did not increase heat resistance in *Drosophila melanogaster* (Bubliy and Loeschcke, 2005).

A number of behavioral and physiological strategies are adopted by insects to avoid or minimize heat injury. Most insects when exposed to high temperatures synthesize heat shock proteins (Parsell and Lindquist, 1993) which act as molecular chaperones and play a useful role in transporting, folding, assembling of denatured or misfolded proteins (Johnston *et al.*, 1998; Sorensen *et al.*, 2003). The heat shock proteins are classified into several groups based on their molecular weight and amino acid composition into Hsp 90, 70, 60, 40 and small Hsps (sHsps) of 12 – 43 kDa (Parsell and Lindquist, 1993). The nature and molecular weight of the heat shock proteins secreted may vary from one animal to another and within the same animal in different tissues and developmental stages (Joplin and Denlinger, 1990). 90 kDa Hsps are moderately conserved in eukaryotes and are usually expressed following exposure to temperature extremes but appear nonessential for thermotolerance (Lindquist and Craig, 1988). The 70 kDa Hsps are highly conserved in

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eukaryotes (Parsell and Lindquist, 1993) and are expressed following most environmental insults. The sHsps contain an α-crystalline domain with molecular weights of 12 – 43 kDa due to variable N-terminal and C-terminal extensions (MacRae, 2000; Taylor and Benjamin, 2005). The synthesis and release of a particular heat shock protein is considered as a direct evidence of the development of thermotolerance (Johnston and Kucey, 1988). Overexpression of Hsps affects normal development (Krebs and Feder, 1997) and reproduction (Krebs and Loeschcke, 1994; Silbermann and Tatar, 2000) and therefore Hsps expression was observed only after exposure to high temperature.

The incidence of heat death in response to imposed heat stress conditions in Pure Mysore (PM) representing multivoltine race and NB4D2 and CSR2 representing bivoltine races was tested and the results were examined in the light of heat shock protein expression.

2 MATERIALS AND METHODS

2.1 Silkworm rearing

Three disease free layings (DFLs) of three silkworm breeds, *viz.* PM, NB4D2 and CSR2, were transferred to a BOD incubator, preset at 25℃ and 80% RH at head pigmentation stage (9th day) of embryonic development. The layings, wrapped in a tissue paper, were transferred to black boxes to synchronize embryonic development. On the following day, the eggs were exposed to a sudden burst of bright light. The light stimulus hastens embryonic development and the young silkworm larvae hatched within 60 min. About 1 500 larvae were transferred to a rearing bed and reared till 5th instar in a previously disinfected rearing house till 4th moult by following standard technique of Dandin *et al.* (2003). Leaf of V-1 mulberry variety was used for feeding the larvae. The standard rearing practice included leaf selection, spacing and bed cleaning besides providing optimal temperature and humidity ambience to the larvae. The larvae, out of 4th moult, were used in the experiment.

2.2 Temperature treatment

Fifth instar larvae on 2nd day and pupae of 3rd day of PM, NB4D2 and CSR2 were exposed to 35, 38 and 40℃ at RH 70% ±5% for 3 h and 6 h. About 50 larvae and pupae in triplicates were used in each temperature treatment. After the heat shock, the larvae and pupae were brought back to normal rearing temperature. Survival of the larvae till spinning of cocoons and of pupae till moth emergence was observed. Probit regression analysis (Finney, 1971) was conducted to analyze the relationship between the mortality and duration of exposure to different temperatures. The Probit analysis was used to obtain the regression equation and determine the feducial limits and LT_{50} values at 35, 38 and 40℃ for the larvae and pupae of PM, NB4D2 and CSR2.

The Probit equation is in the form of $Y = a + bX$.

Where Y is mortality (dependent variable) and X is duration (independent variable), 'a' is a constant being Y intercept, and 'b' regression coefficient of Y on X and reflects the slope of the curve. Feducial limit is the duration required to kill 0 – 100% larvae/pupae. LT_{50} is the time in hours required to kill 50% of the larvae/pupae in an exposure to a high temperature.

2.3 Separation of heat shock proteins by SDS-PAGE

Fifth instar larvae on 2nd day and 3rd day pupae of PM, NB4D2 and CSR2 were exposed to 35, 38℃ for 6 h and 40℃ for 3 h at RH 70% ±5%. About 50 larvae and pupae in triplicates were held in each temperature regime. Ten larvae/pupae were removed for haemolymph collection immediately after each schedule of treatment. The other larvae/pupae were returned to normal room temperature and haemolymph was drawn after 3 h of recovery. Three samples were prepared by pooling the haemolymph of three larvae and used for protein separation.

Haemolymph was collected by cutting the first proleg of the larva in a pre-chilled test tube containing a few crystals of thiourea. Haemolymph from pupae was obtained by inserting a sharp sterilized syringe needle into the first abdominal segment and applying gentle pressure on the thorax and abdomen. The haemolymph was centrifuged at 3 000 g for 10 min at 4℃. The supernatant obtained after centrifugation was used for the estimation of total proteins (Lowry *et al.*, 1951). The protein profiles of the haemolymph of larvae and pupae were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Molecular weight standards ranging from 14.4 to 94 kDa from Pharmacia, USA were also run on each gel and subsequently stained with coomassie brilliant blue. The de-stained gels were placed on a visible trans-illuminator and photographed.

3 RESULTS

3.1 Effect of high temperature on the survival of the silkworms

Temperature tolerance of the larvae and pupae of the three silkworm races held at 35 and 38℃ for 6 h and 40℃ for 3 h was shown as survival percentages (Fig. 1). The larvae of PM showed relatively better survival until 38℃ of ambient temperature. Pupae survived exposure to high temperature better than the larvae.

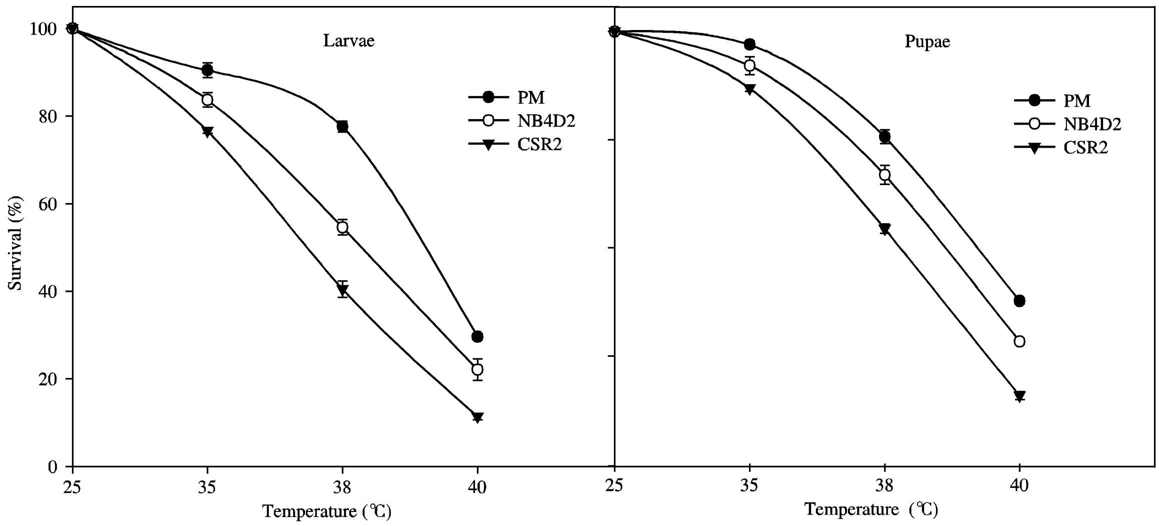

Fig. 1 Effect of high temperature exposure on larval and pupal survival of the silkworm, *Bombyx mori* L.
Each value is the percent mean ± *SD* of three separate observations

3.2 Probit analysis of the effect of high temperature on larvae and pupae of selected silkworm races

Regression coefficients (b_{YX}) of the larvae of NB4D2 and CSR2 were higher than those of PM (Table 1). 'a' and 'b' constants were higher at 40℃ than at 38℃ and 35℃. The feducial limit shrinks at higher temperatures, the range being more at 35℃ and least at 40℃. A significant racial difference was observed in the feducial limits of the larvae. LT_{50} was high at 35℃ and lowest at 40℃. LT_{50} was higher in PM than the two bivoltine races. LT_{50} in NB4D2 was higher than that of CSR2 in the two bivoltine races.

The b_{YX} values of pupae at 38 and 40℃ were relatively lower than the respective b_{YX} values of larvae (Table 1). 'Y' intercept of CSR2 was higher than NB4D2. Also, the 'Y' intercept values in each race were higher at 45℃ than at 40℃. The range of feducial limit in pupae was significantly less than in larvae. The LT_{50} values of pupae at 38 and 40℃ of PM were relatively higher than those of NB4D2 and CSR2. Between the two bivoltine races, NB4D2 showed higher values of LT_{50} than CSR2.

3.3 Protein profiles from heat shocked larvae and pupae

The 90, 70 and 29 kDa protein bands were observed in the haemolymph when the larvae of the three silkworm races were exposed to 38℃ for 6 h (Fig. 2).

But, 3 h exposure to 40℃ resulted in heat shock protein expression in the larval haemolymph in all the three races. However, no significant changes were observed in non-heat shock protein bands at higher temperatures. The 29 kDa protein was not detectable in the haemolymph of bivoltine races after 3 h of recovery whereas it continued expression in the temperature tolerant Pure Mysore. Exposure of pupae to high temperature induced the expression of only 90 and 70 kDa proteins in all the three races (Fig. 3). The profile of non-heat shock proteins was not changed in heat shocked pupae.

Table 1 Probit analysis of larvae and pupae of different races of the silkworm, *Bombyx mori* at selected temperatures

Stage	Races	Treatment temperature	Probit equation	Feducial limit (h)	LT_{50} (h)
Larva	PM	35℃	$Y = 2.98 + 1.13X$	3.56 – 119.6	61.60
		38℃	$Y = 3.11 + 2.68X$	3.02 – 7.39	5.20
		40℃	$Y = 3.57 + 2.78X$	2.86 – 3.71	3.26
	NB4D2	35℃	$Y = 2.77 + 1.33X$	4.12 – 111.57	57.50
		38℃	$Y = 3.44 + 2.42X$	3.57 – 5.40	4.39
		40℃	$Y = 3.82 + 3.67X$	1.91 – 2.28	2.09
	CSR2	35℃	$Y = 2.84 + 1.40X$	4.44 – 76.39	40.40
		38℃	$Y = 3.99 + 1.72X$	3.02 – 4.93	3.86
		40℃	$Y = 4.28 + 3.71X$	1.41 – 1.72	1.56
Pupa	PM	38℃	$Y = 3.07 + 2.70X$	4.08 – 12.51	8.79
		40℃	$Y = 3.85 + 2.64X$	2.42 – 3.07	2.73
	NB4D2	38℃	$Y = 3.45 + 2.47X$	3.48 – 10.13	6.80
		40℃	$Y = 3.96 + 3.44X$	2.24 – 2.68	2.45
	CSR2	38℃	$Y = 4.00 + 1.68X$	3.05 – 8.07	5.56
		40℃	$Y = 4.40 + 2.63X$	1.48 – 1.92	1.69

4 DISCUSSION

Tolerance to high temperature is a complex syndrome involving interaction of a number of genetic and physiological factors. The upper limit of tolerance to temperature is determined by the upper limits of temperature to which the animals were exposed in their natural environment. The three races selected for the

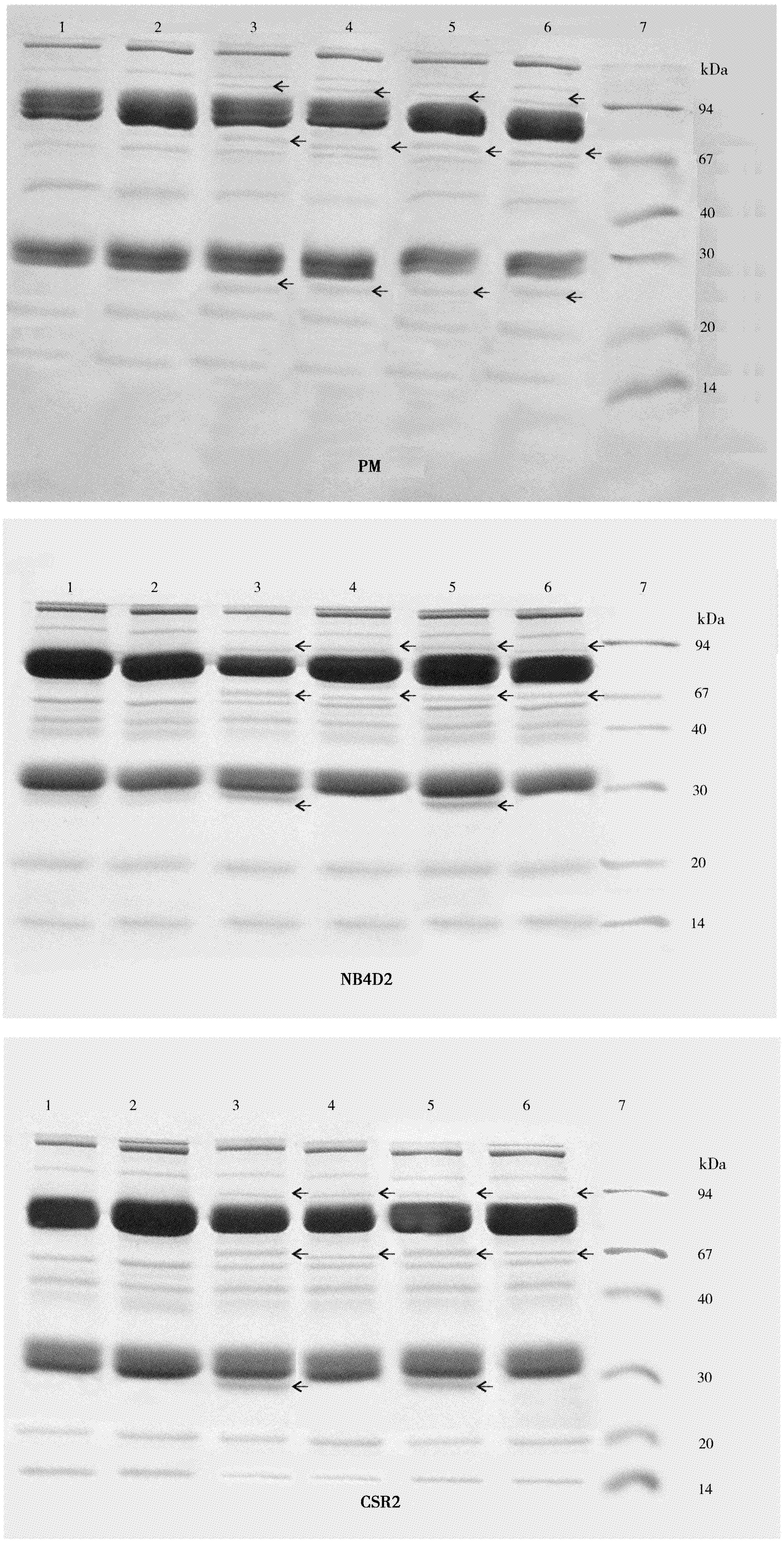

Fig. 2 Haemolymph protein profile of 5th instar larvae of PM, NB4D2 and CSR2 exposed to 35℃, 38℃ for 6 h and 40℃ for 3 h
1: Control (no heat shock); 2: 35℃ heat shock (no recovery time); 3: 38℃ heat shock (no recovery time); 4: Recovery of 3 h after heat shock at 38℃; 5: 40℃ heat shock (no recovery time); 6: Recovery of 3 h after heat shock at 40℃; 7: Marker protein. Heat shock protein bands are indicated by arrows. The same below.

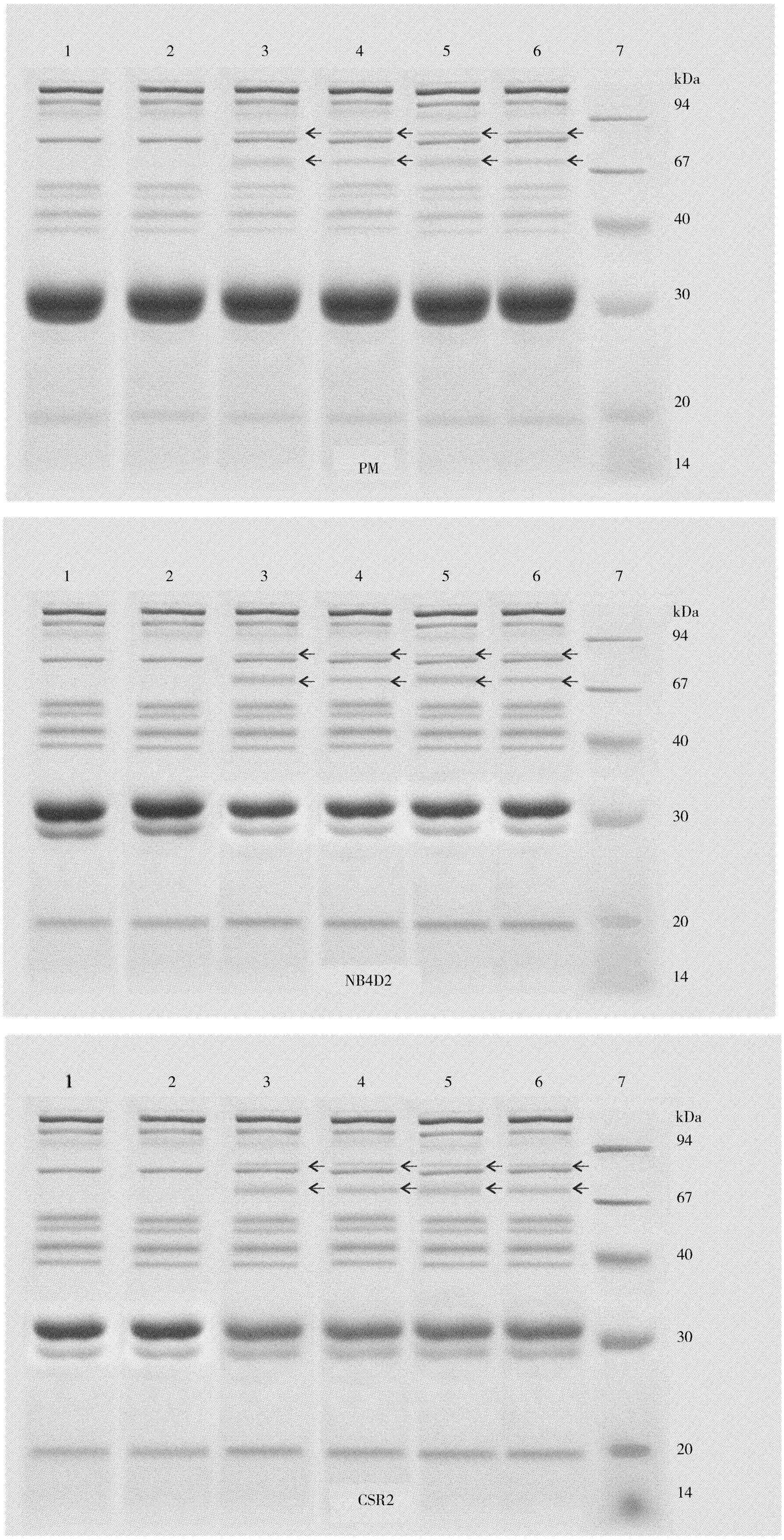

Fig. 3 Protein profile of haemolymph of pupae of PM, NB4D2 and CSR2 exposed to 35℃, 38℃ for 6 h and 40℃ for 3 h

study have different origins and histories of exposure to high temperatures in their natural environment. PM is an indigenous multivoltine race well acclimatized to tropical climates. NB4D2 evolved from a cross between [(Kokko × Seihaku) × (N124 × J124)] is a bivoltine race exposed to tropical climatic conditions though the parents are of temperate origin. CSR2, derived from a cross of Shurei × Shogetsu, is a bivoltine race spinning oval cocoons which is a Chinese race character.

The origin and evolution of the race in its natural environment largely determines the upper limit of its tolerance to high temperature. The genotype and environment interaction over protracted time limits lead to evolution of thermal sensitivity in relation to temperature limits of the habitat. PM reared in tropical climates has scaled up functional thermoregulatory mechanisms and suffers less thermal injury than the bivoltine races at higher temperatures. LT_{50} and b_{YX} values and feducial limits of the three races reveal the relative susceptibilities of the three races to 35, 38 and 40℃ of ambient temperatures. The physical barriers and physiological compensatory mechanisms enable normal function up to certain limits of increase in ambient temperatures. *D. melanogaster* and other mesic species utilize compounds less than 30 carbon atoms in length for epicuticular deposition (Toolson, 1982; Cobb and Jallon, 1990) while *Drosophila* species endemic to the Sonoran desert deposit epicuticular lipids 30 or more carbons (Blomquist *et al.*, 1985) which provide a better barrier to transpiration (Lockey, 1988). The time to kill 50% individuals (LT_{50}) was greatly reduced when the ambient temperature was increased from 35℃ to 38℃ and then to 40℃. Lethality is a function of both temperature and time. The higher the temperature, the shorter the exposure needed to kill the insect. Brief exposure to very high temperatures can cause immediate death, but exposure to a wide range of less severe temperature causes thermal wounding that may be manifested in a number of ways at a later stage of development. The pupae or pharate adults of the flesh fly, *Sarcophaga crassipalpis*, are killed immediately by 2 h exposure to 50℃ (Denlinger *et al.*, 1991) but exposure to a less extreme heat shock, *e. g.* 45℃ for 2 h, the flies die without escaping from the puparium (Yocum *et al.*, 1994) though complete pharate adult development occurs. Pupae show higher temperature tolerance than larvae with no mortality at 35℃ and higher LT_{50} values at 38 and 40℃. Temperature tolerance depends on the efficacy of insulation the epidermal layers provide against external radiation which varies considerably in different stages of insect development. In the flesh fly, *S. crassipalpis*, pharate adults were most tolerant to a 45℃ heat shock, followed by pupae > adult > wandering phase of 3rd instar larvae > feeding phase of 3rd instar larvae (Chen *et al.*, 1991). The hardened puparium is more effective in defending body temperature than the soft larval cuticle against high ambient temperatures. Free living ectotherms are particularly susceptible to the detrimental affects of temperature variation, as their body temperature is determined to a large extent by environmental temperature (Angilletta *et al.*, 2002; Helmuth, 2002).

Environmental temperature affects nearly all biological processes including the structure and function of proteins (Hazel, 1995; Willmer *et al.*, 2004). The fitness character of a race is determined among other things by its ability to preserve the protein structure against heat shock. The protein profiles of the haemolymph include constitutive soluble proteins, haemocyte proteins and tissue proteins released into the haemolymph during circulation. No changes in the constitutive protein profiles of the haemolymph after heat shock treatment were detected. The expression of three heat shock proteins of 90, 70 and 29 kDa was detected in the haemolymph of PM, NB4D2 and CSR2 (Fig. 2). The nature and molecular weight of the heat shock proteins secreted may vary from one animal to another and within the same animal in different tissues and developmental stages (Craig *et al.*, 1993). Joy and Gopinathan (1995) reported the appearance of 93 kDa protein in the haemolymph will impact other somatic and reproductive tissues.

The 70 kDa protein is present constitutively in the fat body and cuticle of C. Nichi and NB4D2 races and on heat shock increased substantially in the fat body in C. Nichi (Joy and Gopinathan, 1995). In the present experiment, Hsp70 secretion is also induced in all the three races in response to 38 and 40℃ heat shock. The 29 kDa sHsp which was not present in haemolymph of control and heat shocked by 35℃ for 6 h, appeared in the haemolymph of silkworms heat shocked at 38℃ for 6 h and 40℃ for 3 h. The 29 kDa protein persisted in the thermotolerant PM after the silkworms were returned to normal temperature after exposure to 38℃ (lane 4) and 40℃ (lane 6) (Fig. 2). But, in the bivoltine races of NB4D2 and CSR2, 29 kDa Hsp which was observed in response to 38 and 40℃ heat shock, disappeared from the haemolymph during the recovery (Fig. 3, lanes 4, 6). Whether the continued presence of this low molecular weight Hsp (29 kDa) in the PM is responsible for its better resistance to high temperatures needs investigation.

In contrast, expression of only 90 and 70 kDa Hsp was observed in pupae heat shocked at 38 and 40℃ for 6 and 3 h, respectively (Fig. 3). The Hsps of the two families appeared immediately after heat shock treatment and their presence was sustained during the recovery period after the pupae were returned to room temperature. No heat shock proteins were observed in pupae held for 6 h at 35℃. Also, Hsps of low molecular weight family were not observed in pupae held at 35 and 38℃ for 6 h and at 40℃ for 3 h. Hsps of low molecular weight were also not observed in the pupae of C. Nichi heat shocked at 41℃ for 1 h (Joy and Gopinathan, 1995). Another significant feature in the haemolymph protein profiles was persistence of non-heat shock haemolymph proteins in all the three races at all the temperature treatments. Lohmann and Riddiford (1992) observed that the lack of repression of non-heat shock protein synthesis in *B. mori*, *Locusta migratoria* (Whyard *et al.*, 1997) and *Manduca sexta* (Fittinghoff and Riddiford, 1990) is an adaptation response observed in the insects inhabiting hot climates.

Thus, higher levels of temperature tolerance were observed in PM than NB4D2 and CSR2 though the parents of all the three races had experienced long-term exposure to hot tropical climates. Lower fecundity and feeding activity in Pure Mysore (Firdose and Srinivasa Reddy, 2006, 2007) and other multivoltine races are useful physiological adaptations to high temperature acclimation. The yellow dung fly, *Scathophaga stercoraria* in warmer climates had smaller ovarioles and testes (Blackenhorn and Henseler, 2005). The reduction in energy support to reproduction increases its availability to heat shock protein synthesis when the organism is confronted with the hot climates. The expression of sHsps was sustained during recovery in PM which favoured thermotolerance to reproduction in the trade off whereas the bivoltine races failed in the continued expression of sHsps genes.

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不同家蚕品系的耐热性特征

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摘要:研究了家蚕 *Bombyx mori* 3 个品系(Pure Mysore,NB4D2 和 CSR2)5 龄幼虫和蛹在不同温度(35,38 和 40℃)下的耐热性,采用 Probit 分析测定了它们在各温度下的 LT_{50}值和置信限。结果表明:多化性品系 Pure Mysore 在高温下的存活率高于两个二化性品系 NB4D2 和 CSR2,而两个二化性品系中 NB4D2 表现出更好的耐热性。家蚕幼虫接触 38℃高温 6 h 和 40℃高温 3 h 后,其血淋巴中出现 90,70 和 29 kDa 的热激蛋白条带。在恢复过程中,NB4D2 和 CSR2 的血淋巴中未见 29 kDa 蛋白条带,而 Pure Mysore 幼虫的血淋巴中 29 kDa 蛋白仍然表达。当幼虫置于高温下时,血淋巴中 90 和 70 kDa 蛋白表达,但是检测不到 29 kDa 蛋白。研究认为热激蛋白表达与热带家蚕不同品系的耐热性以及与同一品系不同发育阶段的耐热性具有相关性。

关键词:家蚕;品系血淋巴;热激蛋白;耐热性

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